Differential effects of five types of antipathogenic plant peptides on model membranes

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Abstract The effects of five antipathogenic plant peptides, wheat α-thionin, potato PTH1 defensin, barley LTP2 lipid transfer protein, and potato tuber DL1 and DL2 defensins, have been tested against phospholipid vesicles (liposomes). Wheat thionin very actively induces aggregation and leakage of negatively charged vesicles. LTP2 displays the same activities, although to a limited extent. Under certain conditions PTH1 and DL2 induce vesicle aggregation, but not leakage. Potato defensin DL1 failed to show any effect on liposomes. The same peptides have been assayed against a plant pathogenic bacterium, both the membrane-active and -inactive compounds having efficient antibacterial action.

Key words: Thionin; Defensin; Lipid transfer protein; Plant pathogen; Biomembrane

1. Introduction

Complex defense mechanisms to control microbial infections have been developed during evolution by both animals and plants. Although defense systems in the two types of organisms have distinctive features, such as the immune response in higher vertebrates or the induction of antimicrobial metabolites in plants, they share some common elements, including the capacity to synthesize antibiotic peptides either constitutively or in response to infection. The characterization of these peptides in plants is being actively pursued and novel antipathogenic peptide families have been recently reported [see ref [1]]. Whether the different peptide families have common or different targets is a relevant question not only with respect to the clarification of the mechanism(s) of action but in connection with the design of novel strategies to engineer plants against pathogens [2].

Thionins, which were the first plant peptides for which an antipathogenic activity had been reported [3,4], have been shown to alter cell membrane permeability [5,6] and to interact with artificial liposomes containing phosphatidylserine [7,8]. Wheat α-thionin contains 45 amino acid residues, with a charge/mass ratio of $+2\times10^{-3}$ [4]. Plant defensins, which are phylogenetically separate but structurally similar to thionins, do not seem to cause substantial membrane permeabilization [6]. Defensin PTH1 from potato, to be used in this study, has 47 amino acid residues, and a charge/mass ratio of $+10^{-3}$ [9]. Little information concerning possible mechanisms of action are available for the more recently described plant peptide families, such as the so-called lipid transfer proteins (LTP), which are extracellular peptides involved in plant defense against pathogens [10–12], and the DL1 and DL2 families of antipathogenic peptides, which may be phylogenetically related to each other and share some common features with snake-venom desintegrins [[13], M. Moreno et al., in preparation]. From the structural point of view, LTP2 from barley is known to contain 90 amino acid residues, with a positive charge/mass ratio of 0.9×10^{-3} [10], while no equivalent data are available for the DL1 and DL2 peptides. In summary, all the peptides tested are water-soluble and, as far as we know, all are positively charged at neutral pH and appear to have a compact globular conformation.

Semisynthetic vesicles surrounded by phospholipid bilayers (liposomes) constitute excellent models for cells and cell membranes in many in vitro studies [14–16]. In particular, the so-called large unilamellar vesicles (LUV), about 100 nm in diameter, obtained by extrusion through polycarbonate membranes [17] are considered to mimick many physical properties of the cell membrane matrix. LUV filled with fluorescent dyes have found extensive application in assessing vesicle leakage caused by detergents [18,19], fusogenic peptides [20] or bacterial toxins [21,22].

In this work, the effects of five types of plant antipathogenic peptides on liposomal preparations of varying lipid composition have been investigated in order to test the hypothesis that these peptides may act at the membrane level in plant pathogenic bacteria. The results indicate that this hypothesis may apply to α-thionin, but most likely not to the other peptides tested.

2. Materials and methods

2.1. Protein purification

A crude thionin preparation was obtained from the endosperm of hexaploid wheat, *Triticum aestivum* L. cv. Candeal, by petroleum-ether extraction and HCl/ethanol precipitation, as previously described [23]. A mixture of genetic variants of wheat thionins was obtained from the crude extract by preparative electrophoresis on 10% polyacrylamide columns (1.5×10 cm) with 0.1 M acetic acid buffer, pH 2.9, at 20 V/cm, essentially as reported [24]. The yield obtained was 50 mg/kg of dry endosperm. Purification of α-thionin was achieved by reverse-phase high-performance liquid chromatography (RP-HPLC), on an Ultrapore C_3 column (1×25 cm; particle 5 μm; pore 300 Å) from Beckman, using H_2O/2-propanol gradients, 0.1% trifluoroacetic acid, with an elution rate of 0.5 ml/min.

To purify all other peptides used in this study, plant material was collected in liquid nitrogen and subjected to a common protocol [9,10,13]. Leaves from barley, *Hordeum vulgare* cv. Bomi, and potato tubers, *Solanum tuberosum* cv. Bintje, were used as sources of the peptides. The frozen material (20 g) was ground to powder in liquid

nitrogen, using a mortar and pestle, and extracted once with 80 ml of buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml of distilled H_2O. The resulting pellet was then extracted with 50 ml of 1.5 M LiCl at 4°C for 1 h, dialyzed against 5 l of H_2O, using a Spectra/Por 6 (MWCO:3000) membrane, and freeze-dried. The extract was subjected to RP-HPLC on an Ultrapore C_3 column (1×25 cm; 5 μm particle; 300 Å pore) from Beckman, using a H_2O/2-propanol gradient, 0.1% trifluoroacetic acid, at 0.5 ml/min. Fractions were collected by hand and freeze-dried. Homogeneity of fractions previously identified as corresponding to the proteins of interest from barley leaves [10] and from potato tubers [[9,13]; Moreno et al., in preparation] was checked by gradient SDS-PAGE and by HPLC, and no detectable traces of contaminant proteins were found [10]. The yields obtained were: 20 mg/kg fresh weight for LTP2, 1.5 mg/kg for the PTH1 defensin, 2 mg/kg and 6 mg/kg for the DL1 and DL2 peptides, respectively. Their antibiotic activity was determined as described below.

2.2. Biological assays

The bacterial pathogen *Clavibacter michiganensis* subsp. *sepedonicus*, strain C5 was from the ETSIA collection (Madrid). Inocula for the inhibition experiments were grown on slanted nutrient agar and removed by shaking the culture with 1 ml of nutrient broth (Oxoid). Bacterial density was measured spectrophotometrically at 600 nm and the suspension adjusted to the appropriate concentration with nutrient broth. Inhibition tests were carried out with the different proteins dissolved in water at the required concentrations. Bacteria were inoculated at a final concentration of 1×10^4 cfu/ml, in sterile microtiter plates at final volumes of 150 μl (100 μl of protein solution +50 μl of nutrient broth, Oxoid). After 2 days of incubation at 28°C, growth was recorded by measuring absorbance at 492–595 nm in an ELISA plate reader. Absorbance increase at 590 nm was measured in an spectrophotometer (1 ml cell) at 15 min and 1 h after adding the proteins to a bacterial suspension of 1×10^6 cfu/ml in water.

2.3. Liposome preparation

Egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) were grade I from Lipid Products (South Nutfield, UK). The appropriate lipids were mixed in organic solvent and evaporated thoroughly. Liposomes (large unilamellar vesicles) were prepared by the extrusion method of Hope et al. [17], using polycarbonate filters 0.1 μm pore size (Nuclepore, Pleasanton). For vesicle aggregation assays, liposomes were prepared in 10 mM HEPES, 200 mM NaCl, 0.5 mM EDTA, pH 7.5 buffer. For assays of vesicle leakage, the buffer contained in addition 25 mM 8-aminonaphthalene-1,3,6,trisulfonic acid (ANTS) and 90 mM *p*-xylene-bis-pyridiniumbromide (DPX) [25]. Non-encapsulated fluorescent probes were eliminated by passing the vesicle suspension through a Sephadex G-75 gel filtration column (Pharmacia, Uppsala) eluted with buffer. Solution osmolarities were checked with an Osmomat 030 instrument (Gonotec, Berlin). Phospholipid concentration was measured according to Bartlett [26].

2.4. Liposome aggregation assay

Vesicle aggregation was routinely followed as an increase in light scattering at 90° in a Shimadzu RF-540 fluorimeter with both monochromators at 520 nm. Occasionally, aggregation was measured as the increase in average particle size, measured by quasi-elastic light scattering (QELS) in a Zetasizer 4 spectrometer (Malvern, UK). For QELS measurements the buffer had been previously filtered trough 0.22 μm filters.

2.5. Assay for leakage of liposomal contents

At high concentrations, (e.g. inside the vesicle) ANTS and DPX form a low-fluorescence complex. Breakdown of the vesicle membrane leads to contents leakage, complex decomposition and high ANTS fluorescence. Thus leakage was assayed by treating the probe-loaded liposomal suspension (0.1 mM) with the appropriate amounts of peptide (usually 10 μg/ml) in a fluorometer cuvette with constant stirring. Changes in fluorescence intensity were recorded vs. time, in a Shimadzu RF-540 spectrofluorometer with excitation and emission wavelengths set at 350 and 520 nm, respectively. 100% release was achieved by adding to the cuvette 5 μl of 10% (w/w) Triton X-100. Leakage was quantified on a percentage basis according to the equation:

$$\%\ \text{release} = \frac{F_f - F_0}{F_{100} - F_0}$$

F_f being the equilibrium value of fluorescence after peptide addition, F_0 the initial fluorescence of the vesicle suspension and F_{100} the fluorescence value after addition of Triton X-100. The measurements were performed at 23°C.

3. Results

3.1. Biological activity of peptides

The capacity to aggregate bacterial cells and the antibiotic activity of the purified antipathogenic peptides were determined for comparative purposes, using the sensitive bacterial pathogen *Clavibacter michiganensis* subsp. *sepedonicus* as test microorganism (Fig. 1). The peptides used in this study were the following: α-thionin (THα) from wheat [27], defensin PTH1 from potato [9], lipid transfer protein LTP2 from barley [10], and two peptides from potato tubers, DL1 and DL2, which may be phylogenetically related to each other and share some common features with snake-venom desintegrins [[13]; M. Moreno et al., in preparation]. All five peptides were able to increase absorbance of bacterial suspensions at 5–20 μM concentrations (Fig. 1A) and to inhibit bacterial growth by 50% (EC_{50}) at 0.1–3 μM concentrations (Fig. 1B). No correlation between the capacity to aggregate bacterial cells and the EC_{50} values was found.

3.2. Liposome assays

Liposomes (LUV) of three lipid compositions, respectively pure PC, PC/PG (1:1 mol ratio) and pure PG, were tested. Both liposome aggregation and vesicle leakage were found to be highly sensitive to lipid composition as well as to the nature of the peptide. These results are summarized in Table 1, while representative examples are shown in Figs. 2 and 3. Wheat thionin (THα) is by far the most active peptide towards liposomal membranes, although its activity is clearly detected only when the lipid is negatively charged, as is PG. In the case of THα there is a certain correlation between aggregation and release (Table 1). This is not the case for PTH1 or DL2 that, although inducing large liposomal aggregation for pure PG vesicles, they fail to cause any release of vesicle contents (Table 1). Lipid transfer protein LTP2 and DL1 were completely inactive towards liposomes at the doses

Table 1
Effect of antipathogenic plant peptides on large unilamellar vesicle aggregation and leakage (release of aqueous contents)

Lipid[a]	Peptides[b]				
	THα	LTP2	PTH1	DL1	DL2
Aggregation[c]					
Pure PC	–	–	–	–	–
PC/PG (1:1)	+++	–	+	–	+
Pure PG	++	–	++	–	++
Release[d]					
Pure PC	–	–	–	–	–
PC/PG (1:1)	80%	–	–	–	–
Pure PG	90%	–	–	–	–

[a]Lipid concentration was 10^{-4} M.
[b]Peptide concentration was 10 μg/ml, corresponding to 1.1–2.0 μM.
[c]Vesicle aggregation is difficult to quantify because of non-Rayleigh scattering effects (see main text). Thus aggregation is represented here semiquantitatively according to the convention: (+++) large increase in turbidity, leading to vesicles >1000 nm in diameter; (++) large increase in turbidity, with detectable increase in size by QELS; (+) increase in turbidity, with only slight increase in size; (–) no effect.
[d]Release of vesicle aqueous contents was quantified as indicated in Section 2.5. Release was measured after 10 min.

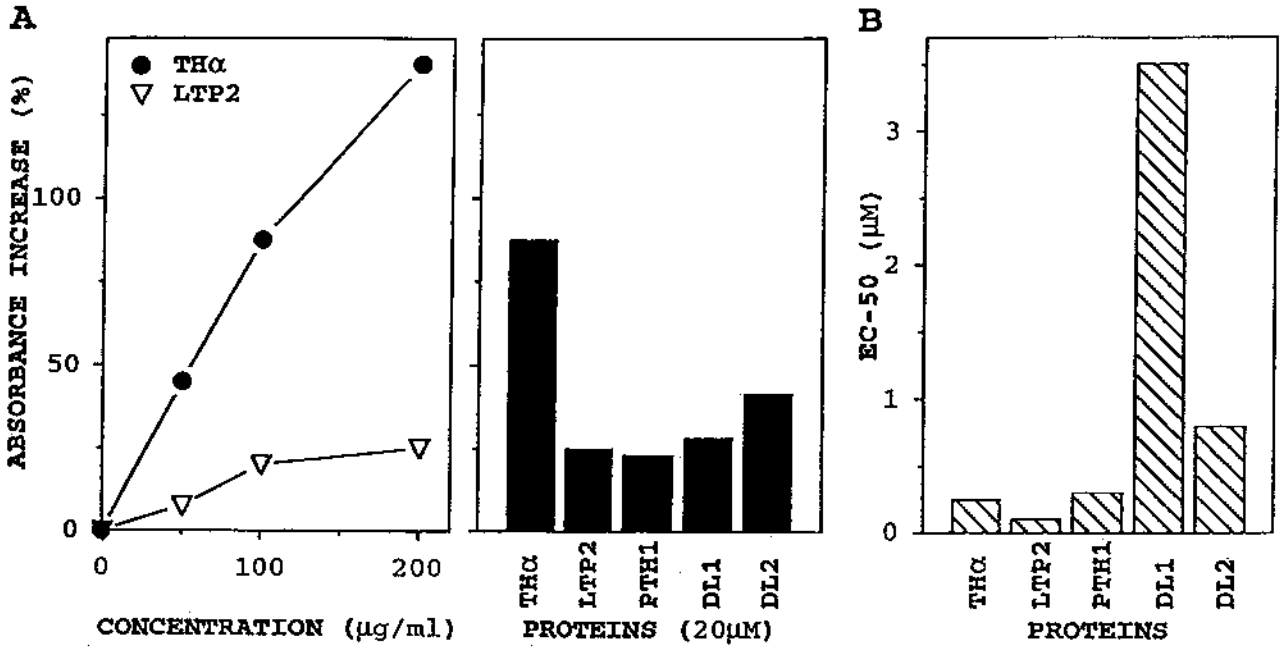

Fig. 1. Effects of different types of plant antipathogenic peptides on the pathogen *Clavibacter michiganensis* subsp. *sepedonicus*. A: Increase (%) of absorbance at 590 nm upon addition of the indicated amounts of the following peptides: α-thionin from wheat, molecular mass ≈5 kDa (THα), lipid transfer protein from barley, molecular mass ≈9 kDa (LTP2), defensin (PTH1), and desintegrin-like peptides (DL1, DL2) from potato. B: Effective concentrations of the five peptides that cause 50% inhibition of growth of the test microorganism.

mentioned above, that correspond to about 10^3 peptide molecules per vesicle.

The data in Figs. 2 and 3 highlight in particular the role of electric charge in the activity of wheat thionin THα. In both cases curve 1 (no activity) corresponds to pure PC liposomes, while curve 2 indicates the thionin effect on vesicles composed of an equimolar mixture of PC and PG. In Fig. 2, curve 2, there is a very fast increase in light scattering, followed by a slower decrease. In fact the decrease is artefactual and due to the large increase in aggregate size (as confirmed by QELS measurements, not shown), to the point that the Rayleigh assumption (i.e., that particle size is smaller than the incident light wavelength) does not longer hold, thus the scattering decreases with increasing particle size [28].

The influence of electrostatic forces on the peptide–vesicle interaction was further tested by comparing the above results, obtained in a buffer containing 200 mM NaCl with studies in which the buffer ionic strength had been lowered by decreasing NaCl to 25 mM. The data are comparatively shown in Table 2. Reducing the ionic strength reduces correspondingly the activity of wheat thionin from the point of view of both liposome aggregation and vesicle leakage. The slight aggregating power of defensin PTH1 disappears completely at low ionic strength. Conversely, the barley lipid transfer protein LTP2, that was inactive at high ionic strength, induces a small degree of aggregation and leakage under low ionic strength conditions. Equally the potato peptide DL2 increases its vesicle aggregation activity at a lower ionic strength, although with this peptide the permeability of phospholipid vesicles remains intact.

4. Discussion

The experiments described above show that five plant peptides, all of them active against the pathogenic bacterium *Clavibacter michiganensis*, display each an individual behaviour against phospholipid vesicles. Wheat α-thionin is very active in inducing liposome aggregation and leakage; potato peptides PTH1 and DL2 induce vesicle aggregation but not leakage; however, the effect of the former decreases with decreasing ionic strength while the opposite happens with DL2. The lipid transfer protein from barley LTP2 has a modest aggregation and leakage-inducing activity, but only at low

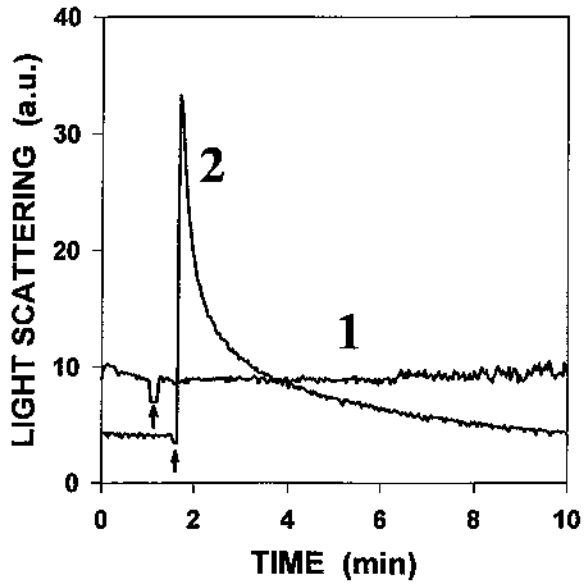

Fig. 2. Liposome aggregation induced by wheat thionin (THα). Aggregation is detected as an increase in light scattering. Liposomal composition: 1, pure PC; 2, PC/PG (1:1). In curve 2 the decrease in light scattering after the initial rapid increase is artefactual (see text). Peptide addition is indicated by arrows. Lipid and peptide concentration were 10^{-4} M and 10^{-6} M, respectively.

ionic strength. Finally, the potato peptide DL1 is totally inactive against liposomes under any conditions tested. These results make relevant a discussion on the interaction of these peptides with lipid bilayers, and on the mechanism of their antibacterial action.

It is commonly accepted that peptide-induced leakage requires the hydrophobic interaction of the peptide with the membrane phospholipid matrix [29–31]. Thus it can be inferred that THα and perhaps LTP2 (Tables 1 and 2) do, under certain conditions, get inserted in the bilayer matrix in a way reminding the integral membrane proteins [32]. In addition to hydrophobic forces other interactions can also be significant. An obvious observation is that virtually no effect is seen with electrically neutral bilayers for any of the peptides tested (Table 1). Peptide insertion is a rather complex process [33], and it is clear that both THα and LTP2 interact electrostatically with the phospholipid headgroups (perhaps neutralizing some peptide cationic residue) before proper insertion occurs. Electrostatic interactions should become more important in a low ionic strength buffer. As seen in Table 2, decreasing ionic strength enhances the effect of DL2 and LTP2, but not that of THα or PTH1, probably as a result of the multiplicity of forces interacting independently. The inhibitory effect on vesicle aggregation induced by THα or PTH1 may be the result of electrostatic repulsion between negatively charged vesicles increasing as a result of lowering the ionic strength, thus making more difficult peptide-induced aggregation. Another complex case of lipid–peptide interaction has been described recently for the large (≈107 kDa) bacterial toxin α-haemolysin [31]. The requirement of negatively charged phospholipids in the liposomes for the peptides to be active may be relevant in connection with the fact that among the major polar lipids in *C. michiganensis* are the negatively charged cardiolipin and phosphatidylglycerol [34].

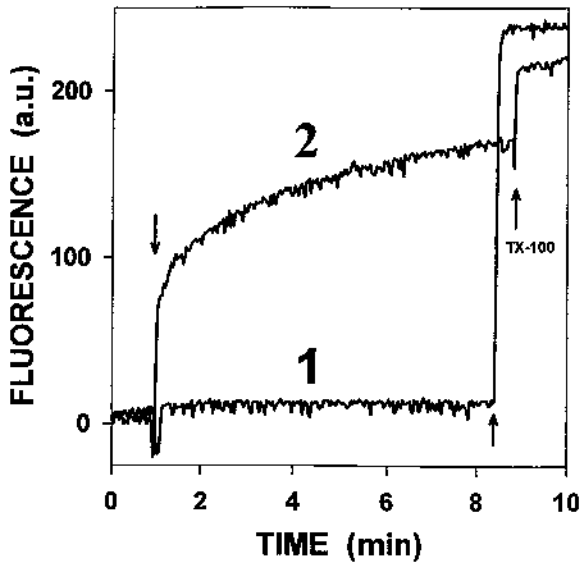

Fig. 3. Kinetics of release of entrapped solutes from liposomes by the action of wheat thionin (THα). Leakage is marked by an increase in fluorescence intensity. Liposomal composition: 1, pure PC; 2, PC/PG (1:1). The downward arrow indicates the time of peptide addition; the upward arrows show the addition of Triton X-100. Curve 2 has been corrected for a small (≈3%) influence of scattering on the fluorescence intensity. Lipid and peptide concentration were 10^{-4} M and 10^{-6} M, respectively.

Table 2
The effect of ionic strength on the membrane lytic activities of antipathogenic plant peptides

Peptide	Aggregation (200 mM)	(25 mM)	Release (200 mM)	(25 mM)
THα	+++	+	80%	17%
LTP2	–	+	–	21%
PTH1	+	–	–	–
DL1	–	–	–	–
DL2	+	++	–	–

Experimental details and explanation of signs are as in Table 1.
The effects of the various peptides were tested against PC/PG (1:1) liposomes, in buffer containing either 200 mM or 25 mM NaCl.

With respect to the mechanism of action of the antibacterial peptides under study, considering the various peptide effects, i.e., bacterial aggregation, bacterial growth inhibition, liposome aggregation and liposome leakage, there is no clear correlation among all four effects observed for any of the peptides. It can be seen, however, that wheat thionin is a good bacterial aggregant (Fig. 1A), its EC_{50} is relatively low (Fig. 1B) and it certainly perturbs membrane structure (Table 1). The present data would support the cell membrane as a target (or one of the targets) for its action, in agreement with previous studies using cell membranes [5,6]. The potato peptide DL1 represents the opposite case; it has a low bacterial aggregation activity (Fig. 1A) and no effect on lipid membranes (Table 1). Membranes are not a likely target for the antibacterial action of DL1. The remaining peptides represent intermediate cases but, in view of the results in Tables 1 and 2, only the barley lipid transfer protein LTP2 may act, at least partially, through impairment of the membrane permeability barrier.

In summary, of the five antipathogenic plant peptides examined here the data support that one (THα) or at most two (THα and LTP2) may act at the membrane level, while the effect of a third one (DL1) is unlikely to occur at that level. It can be concluded that, in spite of functional, structural or phylogenetic similarities, these peptides may have very different mechanisms of antipathogenic action.

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